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Acute Depression of Mitochondrial Protein Synthesis during Anoxia

CONTRIBUTIONS OF OXYGEN SENSING, MATRIX ACIDIFICATION, AND REDOX STATE*

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Mitochondrial protein synthesis is acutely depressed during anoxia-induced quiescence in embryos of *Artemia franciscana*. Oxygen deprivation is accompanied *in vivo* by a dramatic drop in extramitochondrial pH, and both of these alterations strongly inhibit protein synthesis in isolated mitochondria. Here we show that the oxygen dependence is not explained simply by blockage of the electron transport chain or by the increased redox state. Whereas oxygen deprivation substantially depressed protein synthesis within 5 min and resulted in a 77% reduction after 1 h, aerobic incubations with saturating concentrations of cyanide or antimycin A had little effect during the first 20 min and only a modest effect after 1 h (36 and 20% reductions, respectively). Yet the mitochondrial NAD(P)H pools were fully reduced after 2–3 min with all three treatments. This cyanide- and antimycin-insensitive but hypoxia-sensitive pattern of protein synthesis depression suggests the presence of a molecular oxygen sensor within the mitochondrion. Second, we show for the first time that acidification of extramitochondrial pH exerts inhibition on protein synthesis specifically through changes in matrix pH. Matrix pH was 8.2 during protein synthesis assays performed at the extramitochondrial pH optimum of 7.5. When this proton gradient was abolished with nigericin, the extramitochondrial pH optimum for protein synthesis displayed an alkaline shift of ~0.7 pH unit. These data suggest the presence of proton-sensitive translational components within the mitochondrion.

Exposure of brine shrimp embryos (*Artemia franciscana*) to anoxia results in the largest acidification of intracellular pH ever reported for eukaryotic cells, with pH falling from ≥ 7.7 to ≤ 6.8 in the first 20 min (1, 2) and to ≤ 6.3 upon longer exposure (1). This pH transient is thought to play a dominant role in the induction of a reversible quiescent state under anoxia (3), which can last in excess of 2 years (4). The acidification of intracellular pH has been strongly implicated in the arrest of both catabolic (Refs. 5–7; reviewed in Ref. 8) and anabolic processes in the cytoplasm (9–12). Similarly, protein synthesis in mitochondria from these embryos is also reduced markedly (up to 90%) in response to anoxia and acidic pH (13). In the

present study, we examined mechanisms that mediate the inhibitory effects of both oxygen deprivation and extramitochondrial pH acidification on mitochondrial protein synthesis.

In *A. franciscana* mitochondria, the depression of protein synthesis in response to either oxygen deprivation (13) and/or pH acidification (14) appears to be global in that no qualitative differences are detectable in the array of translation products synthesized in response to these factors (13). Furthermore, this depression is likely exerted post-transcriptionally; levels of selected mitochondrial mRNA (e.g. *COXI*) do not decrease during short term (6 h) anoxia *in vivo* (15), yet mitochondrial protein synthesis *in vitro* is substantially depressed after 5 min of anoxia (13). The depression of mitochondrial protein synthesis in yeast observed after longer term (hours) exposure to anoxia is also thought to be exerted, in part, post-transcriptionally (reviewed in Refs. 16 and 17). However, the precise mechanisms that mediate the acute effects of either oxygen deprivation or extramitochondrial pH acidification on translation within the mitochondrion are not known.

In isolated mitochondria from *A. franciscana* embryos, matrix ATP:ADP and GTP:GDP ratios decline with increasing time under anoxia at constant pH (13). However, the addition of ATP (1 mM) at the onset of anoxia stabilizes the ATP:ADP ratio at aerobic values but does not rescue protein synthesis (13). Thus, the anoxia-induced decrease in the matrix ATP:ADP ratio does not cause the inhibition of protein synthesis. Because we were unsuccessful in stabilizing the GTP:GDP ratio under anoxia (13), the reduction in this ratio could contribute to the depression of protein synthesis during anoxia by altering rates of translational initiation and/or elongation. However, the fact that protein synthesis is rapidly depressed before purine nucleotide levels substantially change suggests that other factors are involved. For example, anoxia-induced changes in redox potential (see discussions in Refs. 18 and 19) are known to alter both transcriptional and translational rates in some organisms. Here, we have differentiated between the effects of oxygen limitation *per se* and redox alterations by comparing rates of mitochondrial protein synthesis under anoxia with aerobic rates in the presence of electron transport inhibitors (cyanide or antimycin A). Our data support the conclusion that redox changes caused by blockage of the electron transport chain do not explain the majority of inhibition in protein synthesis for isolated mitochondria under anoxia. Rather the data suggest the presence of a sensor for molecular oxygen (or oxygen byproducts) within the mitochondrion.

Matrix purine nucleotides do not change in response to pH during aerobic incubations of *A. franciscana* mitochondria (13), and therefore alterations in the mitochondrial energy status cannot account for the observed depression of protein synthesis at low pH. In addition, alterations in pH do not appear to inhibit the import of amino acids (13). One hypothesis explored in the present study is that protons have a direct effect on

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components of the mitochondrial translational machinery through changes in matrix pH. The pH sensitivity of translational components in the cytoplasm of these embryos has previously been documented (11), and initial studies indicated that substantial changes in matrix pH do occur in response to extramitochondrial pH (13). Here we have extended our measurements of matrix pH and examined the pH sensitivity of protein synthesis in the presence of nigericin, a H^+/K^+ exchanger that abolishes mitochondrial ΔpH (20). We predicted that if matrix pH is directly affecting protein synthesis, then an alkaline shift in the extramitochondrial pH optimum should occur with nigericin that is similar in magnitude to the ΔpH in the absence of nigericin. This prediction is supported by the data presented.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were of the highest purity commercially available and were obtained from Sigma unless otherwise stated. Sucrose was purchased from Pfanstiehl (Waukegan, IL), and [3H]leucine was obtained from DuPont NEN. The acetoxyethyl ester of BCECF¹ was obtained from Molecular Probes (Eugene, OR). Encysted embryos of *A. franciscana* (Optima Grade) were obtained from Sanders Brine Shrimp (Ogden, UT).

Measurements of Mitochondrial Protein Synthesis—Mitochondria were isolated from dechorionated *A. franciscana* embryos by differential centrifugation (14) in mitochondrial isolation medium consisting of 500 mM sucrose, 150 mM KCl, 20 mM HEPES (pH 7.5 at 25 °C), 1 mM EGTA, and 0.5% (w/v) fatty-acid-free bovine serum albumin (fraction V, Sigma). To assess rates of mitochondrial protein synthesis, [3H]leucine incorporation into trichloroacetic acid-insoluble protein was quantified using a glass-filter-based assay (14). Before the assays were initiated, mitochondria (~1.0 mg protein/ml) were preincubated with 5 mM succinate, 1.0 mM ADP, and 10 mM KH_2PO_4 for 10 min at room temperature (22–23 °C) in protein synthesis medium consisting of 500 mM sucrose, 150 mM KCl, 20 mM HEPES, 4 mM $MgCl_2$, 1 mM EGTA, 0.5% bovine serum albumin, and 0.1 mg/ml cycloheximide (14). This period of time is sufficient for the complete phosphorylation of added ADP (2). Assays were then initiated by the addition of [3H]leucine (60 μM ; specific activity, 10 $\mu Ci/nmol$) and a mixture of 19 unlabeled amino acids (0.3 mM of each). At designated time points, aliquots were removed and [3H]leucine incorporation was quantified. Antimycin A (Sigma A8674, a mixture of A_1 and A_2) was dissolved in 100% ethanol immediately before use. The final ethanol concentration in the antimycin A assays was $\leq 4\%$, and this amount was added to all other treatments to allow direct comparisons. Protein synthesis assays under anoxia (nominally oxygen-free conditions) were performed in a N_2 -equilibrated glovebox as described previously (13).

Mitochondrial Respiration with Electron Transport Inhibitors—Measurements of mitochondrial respiration were performed as described previously (2) with minor modifications. In brief, 100 μl of a mitochondrial suspension (2–3 mg of protein) were added to 1.4 ml of respiration medium containing rotenone (2). Oxygen uptake was measured with Strathkelvin model 1302 oxygen electrodes in RC350 glass respiration chambers. After temperature equilibration (25 °C) of the mitochondria in respiration medium, 5 mM succinate was added to the chamber, and state 2 respiration was digitally recorded for several minutes. KCN or antimycin A was then added, and the mixture was incubated for 5 min, after which 150 μM ADP was introduced and state 3 respiration was recorded.

Respiration data were analyzed with DatGraf software (Oroboros, Innsbruck, Austria). Oxygen concentration in the chamber (c_{O_2} , nmol O_2/ml) was calculated from pO_2 measurements based on O_2 solubility in the respiration medium at 25 °C and ambient barometric pressure. Oxygen flux (J_{O_2} , pmol $O_2 \cdot s^{-1} \cdot ml^{-1}$) was calculated as the time derivative of c_{O_2} . Corrections were made for 1) consumption of oxygen by the electrode and back diffusion of oxygen into the chamber in the absence of mitochondria, 2) the exponential time constant of the oxygen sensor, and 3) transient changes in c_{O_2} resulting from the injection of solutions. K_i values for KCN and antimycin A were calculated with the WILMAN 4 kinetics package (Michigan State University) according to the methods of Wilkinson (21).

Measurements of Matrix Redox State—Changes in the matrix ox-

idoreduction state of NADH + NADPH were assessed by measuring endogenous fluorescence of mitochondrial suspensions. In brief, 3 ml of mitochondrial suspension (~2 mg protein/ml of protein synthesis medium) were added to a 4-ml quartz cuvette and constantly stirred at 25 °C. Emission fluorescence (455 nm) was monitored with an Aminco SLM-48000 spectrofluorometer using an excitation wavelength of 350 nm and 4-nm slit widths. For each assay, the value for maximal reduction and oxidation of the matrix NAD(P) pool was quantified by titration with 3-hydroxybutyrate and acetoacetate, respectively, in the presence of rotenone as described previously (22). The data are presented as a percentage of maximal reduction as achieved with 20 mM 3-hydroxybutyrate. For the anoxic assays, mitochondrial suspensions were equilibrated with 100% N_2 in a glovebox, and then 1 ml of mineral oil was layered over the surface before the cuvettes were removed from the nitrogen environment.

Because 3-hydroxybutyrate and acetoacetate were used to titrate mitochondrial redox state, it was important to verify the presence of β -hydroxybutyrate dehydrogenase activity in *A. franciscana* embryos. Dechorionated embryos were homogenized in 3 volumes of medium consisting of 50 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 1 $\mu g/ml$ soybean trypsin inhibitor and centrifuged at $1000 \times g$ for 10 min. 30 μl of supernatant was assayed at 340 nm in a 1-ml reaction mixture containing 150 mM Tris-HCl (pH 8.0), 1.9 mM β -NAD, and 27 mM 3-hydroxybutyrate. β -Hydroxybutyrate dehydrogenase activity was 0.26 ± 0.01 units/g wet tissue mass (mean \pm S.E., $n = 6$).

Measurements of Matrix pH—Matrix pH was measured with the pH-sensitive fluorescent dye BCECF as described previously (13). In brief, mitochondria (~25 mg of protein/ml of mitochondrial isolation medium) were incubated with 10 μM BCECF/acetoxyethyl for 20 min at 25 °C, diluted 10-fold with ice-cold mitochondrial isolation medium, and centrifuged at $9000 \times g$ for 10 min to wash away unhydrolyzed dye. The mitochondria were resuspended with mitochondrial isolation medium to obtain the original stock concentration and stored on ice until use. The ratio of fluorescence emission at 535 nm/450 nm was monitored at the excitation λ_{max} of 504 nm with an Aminco SLM-48000 spectrofluorometer using 4-nm slit widths and a temperature-controlled, stirred cell holder maintained at 25 °C. Matrix pH was estimated from this ratio using a calibration curve generated with detergent-lysed mitochondria (13).

Statistical Analyses—Analysis of covariance was used to compare rates of mitochondrial protein synthesis among various treatments, and analysis of variance was used to compare the matrix oxidoreduction state of NAD(P)H after arcsine transformation of percentage data (SAS Institute, Cary, NC). Adjustments of α values (Bonferroni) were made when multiple comparisons were performed.

RESULTS

Influence of Anoxia, KCN, and Antimycin A on Mitochondrial Protein Synthesis—Exposure of isolated mitochondria to anoxia at a constant extramitochondrial pH promotes a rapid and striking inhibition of mitochondrial protein synthesis that is observable within 5 min (Fig. 1). The incorporation of [3H]leucine was depressed by 77% after 60 min of oxygen deprivation relative to the values for aerobic controls (Fig. 1). To determine whether the effect of anoxia was due simply to blockage of the electron transport chain, aerobic mitochondria were exposed to KCN and to antimycin A. The inhibitor concentrations initially chosen were based on the ability to fully block state 3 and state 4 respiration in isolated mitochondria (Table I); these levels were further increased to obtain maximal inhibition of protein synthesis (Table II). K_i values for both inhibitors were comparable with those reported for mitochondria from other sources (23). Both of these respiratory inhibitors depressed protein synthesis in a concentration-dependent (Table II) and time-dependent (Fig. 1) manner. However, the effects were surprisingly small in comparison to those observed with anoxia. At concentrations of KCN and antimycin A greater than or equal to those eliciting complete respiratory inhibition (500 μM and 100 μM , respectively), total [3H]leucine incorporation was lowered only by 36% after 60 min with KCN, and inhibition by antimycin A was even less (20%) (Fig. 1). Increasing the cyanide concentration from 500 μM to 5 mM did

¹The abbreviation used is: BCECF, 2'-7'-bis-(2-carboxyethyl)-5-carboxyfluorescein.

not result in significantly greater inhibition of protein synthesis ($p > 0.83$, data not shown). At all time points examined, protein synthesis rates under anoxia were significantly lower ($p \leq 0.01$) than those with either of the electron transport inhibitors.

Matrix Redox State—To ascertain whether the substantially greater inhibition of protein synthesis by anoxia (compared with that seen with electron transport inhibitors) was due to differences in redox state among these treatments, endogenous fluorescence of mitochondria was used to follow changes in total redox state of the NAD and NADP pools in the matrix. Under aerobic conditions, the degree of reduction in the matrix was 69% of the maximum value obtained by titration with 3-hydroxybutyrate, consistent with previous reports (24). As expected, exposure of mitochondria to anoxia caused a substantial increase in the percentage of reduction (Table III). Importantly, both of the electron transport inhibitors promoted an increase in the redox state that was statistically the same as that elicited by anoxia ($p > 0.05$). After the addition of either 500 μM KCN or 100 μM antimycin A, the redox state of the matrix rapidly became more reduced, and within 2–3 min it was stable for the duration of the 1-h assays (data not shown). Thus the differences in inhibition of protein synthesis by anoxia, KCN, and antimycin A cannot be explained by differences in redox state.

Matrix pH as a Function of Extramitochondrial pH—To verify the ability of BCECF to detect changes in matrix pH under the experimental conditions used, we sequentially titrated mitochondrial suspensions with respiratory substrates that are known to alter matrix pH (25). Following the addition of suc-

cinatate, matrix pH rapidly increased and began to plateau within 10 min (Fig. 2). This increase in matrix pH is consistent with proton extrusion from the matrix during state 2 respiration. The addition of KH_2PO_4 resulted in a rapid decline in matrix pH, which would be predicted from the mitochondrial import of P_i via the H^+/P_i symport (20). The addition of ADP resulted in a decrease in matrix pH that was maintained throughout the duration of state 3 respiration (determined by parallel assays of oxygen consumption). The decrease in matrix pH promoted by ADP is consistent with proton entry through ATP synthase during state 3 respiration. Following state 3 respiration, matrix pH increased and eventually plateaued after several minutes (data not shown). Thus, BCECF serves as a reliable indicator of matrix pH for *A. franciscana* mitochondria.

Fig. 3 shows the steady-state values of matrix pH (A) and ΔpH (B) as a function of extramitochondrial pH values that span the relevant physiological range (pH 6.3–7.9). Interestingly, both matrix pH and ΔpH decreased with decreasing extramitochondrial pH. Others (26–28) have reported a modest increase in ΔpH with decreasing extramitochondrial pH over a similar pH range. At present, the explanation for this difference in *A. franciscana* mitochondria is not clear. However, in the context of the anoxic embryo *in vivo*, a key point supported by Fig. 3 is that as cytoplasmic pH plummets, the matrix pH would apparently experience an acidification of even larger magnitude.

To provide a baseline for subsequent experiments with nigericin (described below), it was important to quantify precisely the ΔpH for isolated mitochondria under actual conditions of protein synthesis. The temporal profile of change in matrix pH during a 1-h protein synthesis assay at an extramitochondrial pH of 7.5 is shown in Fig. 4. All components required for oxidative phosphorylation and protein synthesis were supplied together at time 0. Matrix pH rapidly increased during the first 5 min and thereafter remained relatively constant to the end of the 1-h assay. Steady-state values for matrix pH and ΔpH during protein synthesis assays at three different extramitochondrial pH values are given in Table IV. The initial change (0–5 min) in matrix pH during these assays was not related to the occurrence of protein synthesis *per se* because similar changes in matrix pH were observed in the presence of puromycin (Fig. 4, filled symbols). Rather, the rapid alkalization in matrix pH is likely the result of oxidative phosphorylation, because this same initial pattern was observed during a 10-min preincubation with succinate and ADP prior to initiation of the protein synthesis assay (data not shown).

Finally, it is appropriate to note that at an extramitochondrial pH of 7.5 matrix pH did not significantly change during a 3-h exposure of isolated mitochondria to oxygen deprivation (Table V). We find this constancy of the pH gradient remarkable in the absence of active proton translocation via the electron transport chain. Yet by comparison, Andersson *et al.* (29) showed that the ΔpH was maintained for at least 30 min under anoxia in rat liver mitochondria. Both observations suggest that mechanisms exist to depress proton leakage across the inner membrane during anoxia.

pH Profiles for Protein Synthesis in the Presence of Nigeri-

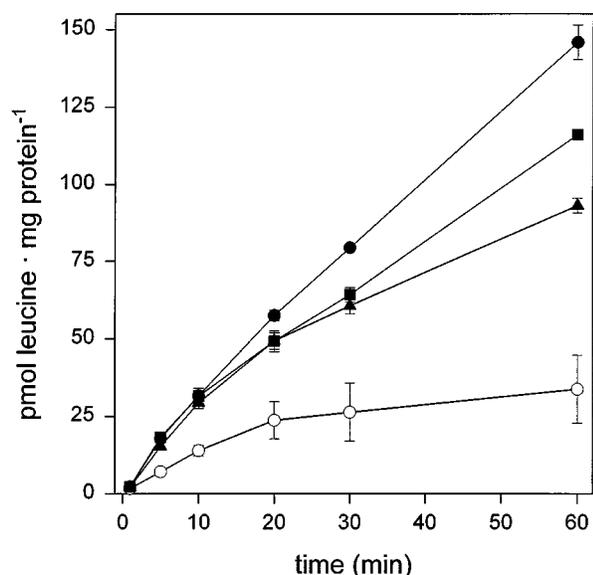


FIG. 1. Comparison of the effects of antimycin A, KCN, and anoxia on protein synthesis in isolated mitochondria from *A. franciscana* embryos. Filled circles are aerobic controls, squares are assays containing 100 μM antimycin A, triangles are assays containing 500 μM KCN, and open circles are anoxic assays. The data are the means \pm S.E. for at least three independent assays from multiple mitochondrial preparations at each time point.

TABLE I
Sensitivity of respiration in *A. franciscana* mitochondria to inhibitors of electron transport

Inhibitor	State 3 respiration		State 4 respiration	
	K_i	Concentration for maximum inhibition	K_i	Concentration for maximum inhibition
KCN	1.74 ± 0.20 ($n = 10$) ^a	500	5.04 ± 1.24 ($n = 10$)	500
Antimycin A	0.017 ± 0.007 ($n = 9$)	1	0.124 ± 0.029 ($n = 9$)	1

^a All values are given in micromolar concentrations, and K_i values are expressed as the means \pm S.E.

TABLE II
Titration of mitochondrial protein synthesis with electron transport inhibitors

Inhibitor	Concentration	Leucine incorporation	% of control
	μM	$\text{pmol} \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$	
KCN	5	132 ± 9^a	90.4
	50	114 ± 3	78.1
	500	93 ± 2	63.7
Antimycin A	0.1	132 ± 1	90.4
	1	122 ± 1	83.6
	10	122 ± 2	83.6
	100	116 ± 1	79.6

^a Values are expressed as the means \pm S.E. ($n = 3$).

TABLE III
Influence of anoxia, KCN, and antimycin A on the matrix redox state of NAD(P) in isolated mitochondria from *A. franciscana* embryos

Treatment (60-min duration)	Percent of maximal reduction ^a
Aerobic control	69 ± 11^b
Anoxia	98 ± 2
KCN (500 μM)	94 ± 5
Antimycin A (100 μM)	92 ± 9

^a Values for maximally reduced mitochondria were determined by titration with 3-hydroxybutyrate.

^b Values are expressed as the means \pm S.E. ($n = 4$).

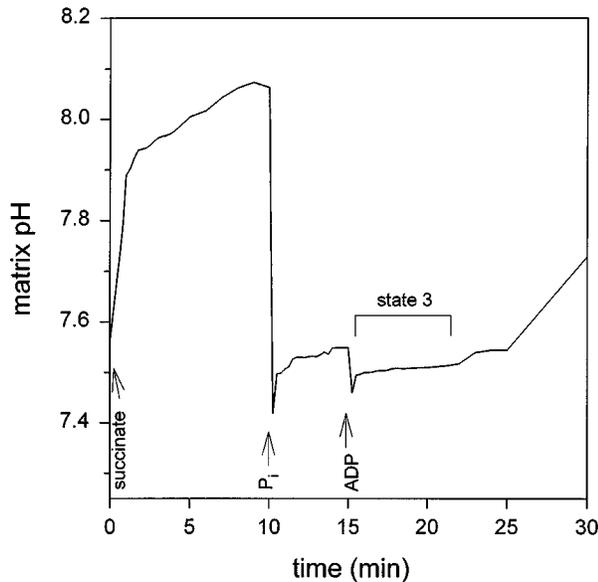


FIG. 2. Change in matrix pH during transitions between state 2 and state 3 respiration in isolated mitochondria from *A. franciscana* embryos. The pH of the medium was 7.4. 5 mM succinate was added at time 0, 10 mM KH_2PO_4 was added at 10 min, and 125 μM ADP was added at 15 min. The duration of state 3 respiration was determined in parallel assays of oxygen consumption and is indicated on the figure.

cin—To address whether the strong dependence of mitochondrial protein synthesis on extramitochondrial pH (reduced 80% by dropping pH from 7.5 to 6.8 (14)) could be a direct result of changes in matrix pH, the pH optimum for protein synthesis was determined in the presence and the absence of nigericin. Maximum dissipation of the proton gradient was observed with 100 μM nigericin (data not shown). With this concentration of nigericin, the ΔpH was not statistically different from zero ($p \geq 0.09$) at any point during protein synthesis assays performed at pH values between 6.7 and 8.1 (Table VI). The pH optimum for protein synthesis showed an alkaline shift from approximately 7.5 in the absence of nigericin to pH 8.2 with 100 μM nigericin (Fig. 5). Considering that steady-state matrix pH was 8.2 at the optimal extramitochondrial pH of 7.5 in control mitochondria

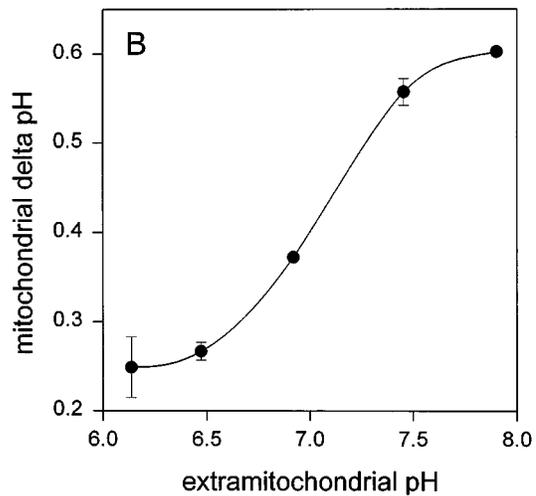
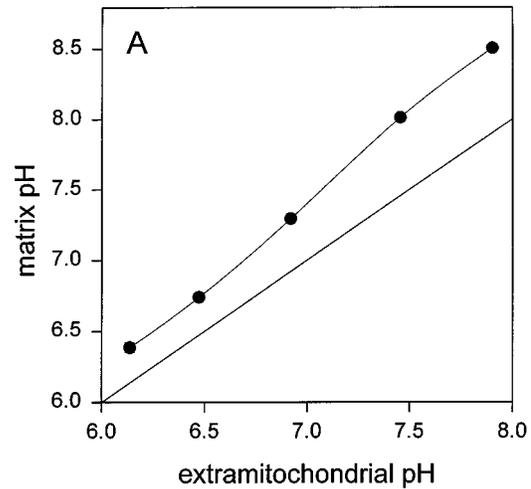


FIG. 3. Matrix pH (A) and ΔpH (B) as a function of extramitochondrial pH in isolated mitochondria from *A. franciscana* embryos. The line of unity is indicated in A. BCECF-loaded mitochondria were placed in respiration medium, and matrix pH was measured under steady-state conditions. The data are the means \pm S.E. for three independent determinations at each pH.

(Table IV), the 0.7-unit shift in the extramitochondrial pH optimum seen upon abolishing the ΔpH provides convincing evidence that mitochondrial protein synthesis responds to matrix pH and not to extramitochondrial pH or ΔpH .

DISCUSSION

This study has revealed two previously undescribed features of mitochondrial bioenergetics that concern the mechanisms by which oxygen deprivation and acidic extramitochondrial pH acutely depress organellar protein synthesis. First, the rapid reduction of protein synthesis by $\sim 80\%$ that occurs when oxygen is removed cannot be explained by blockage of the electron transport chain *per se* or by the associated change in redox state. Rather, as developed below, a case is made for direct signaling through the removal of molecular oxygen and/or oxygen byproducts that is mediated by an oxygen sensor. Such mechanisms for *acutely* down-regulating mitochondrial protein synthesis under anoxia are distinct from other identified controls of gene expression that are operative during longer term (hours) anoxia in yeast (reviewed in Refs. 17, 30, and 31). Second, our results indicate that the pronounced sensitivity of mitochondrial protein synthesis to extramitochondrial pH is a direct result of associated changes in the matrix pH, as opposed to alterations in the ΔpH or the import of amino acids for

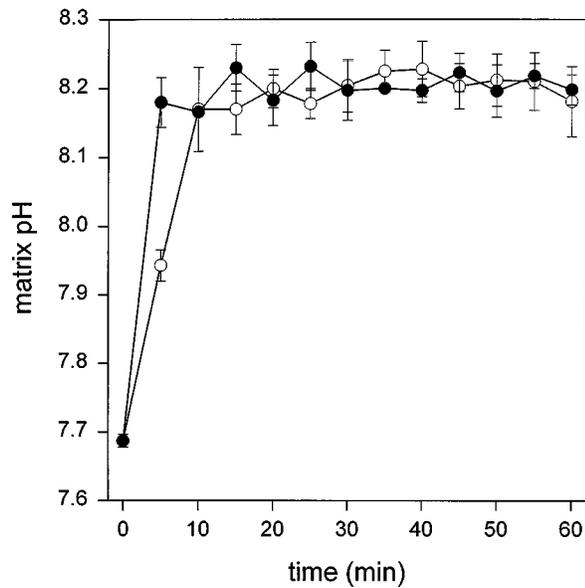


FIG. 4. Matrix pH in mitochondria from *A. franciscana* embryos during protein synthesis assays at pH 7.5. Open symbols are assays performed in the absence of puromycin, and closed symbols are assays performed with puromycin (0.4 mg/ml). The data are the means \pm S.E. for three independent determinations at each time point.

TABLE IV

Matrix pH and Δ pH under conditions of active protein synthesis in *A. franciscana* mitochondria

Extramitochondrial pH	Matrix pH	Δ pH
6.79	7.10 \pm 0.02 ^a	0.31
7.50	8.20 \pm 0.04	0.70
7.78	8.46 \pm 0.03	0.68

^a Values are expressed as the means \pm S.E. ($n = 3$).

TABLE V

Matrix pH and Δ pH as a function of time under anoxia in mitochondria isolated from *A. franciscana* embryos

Time under anoxia	Matrix pH ^a	Δ pH
<i>min</i>		
30	8.09 \pm 0.01	0.65
60	8.09 \pm 0.01	0.65
90	8.10 \pm 0.01	0.66
120	8.14 \pm 0.04	0.70
150	8.12 \pm 0.02	0.68
180	8.11 \pm 0.03	0.67

^a Matrix pH was measured at an extramitochondrial pH of 7.5, and the values are expressed as the means \pm S.E. ($n = 3$). At all time points examined, anoxic values of matrix pH were not significantly different from those of aerobic controls ($p \geq 0.54$).

example. Sensitivity of cytoplasmic protein synthesis to pH has been previously documented for *A. franciscana* embryos (11), as well as for other cell types (32–34), yet a direct proton effect on biosynthesis within the mitochondrion has never been reported. This latter observation is of particular interest in the context of cells that experience transients in cytoplasmic pH, because it suggests that one signal can simultaneously serve to down-regulate biosynthesis in two cellular compartments. Furthermore, the result implicates the presence of proton-sensitive components within the mitochondrial translational machinery.

Oxygen Deprivation and Acute Inhibition of Protein Synthesis—Because anoxia-induced changes in redox potential are known to affect gene expression at both translational and transcriptional levels in some organisms (18, 35), we tested this possibility for isolated mitochondria by adding saturating levels of cyanide and antimycin A. Cyanide blocks electron transport by holding the heme a_3 of cytochrome *c* oxidase in the

TABLE VI
Effect of nigericin on mitochondrial Δ pH as a function of extramitochondrial pH in isolated mitochondria from *A. franciscana* embryos

BCECF-loaded mitochondria were preincubated with ADP and succinate prior to the addition of nigericin (100 μ M) and initiation of the protein synthesis assay. The data are the means \pm S.E. for pooled time points (20, 40, and 60 min) of three independent assays at each pH.

Extramitochondrial pH	Δ pH
6.71	0.01 \pm 0.01
6.91	0.01 \pm 0.01
7.15	-0.03 \pm 0.01
7.40	0.06 \pm 0.02
7.82	0.05 \pm 0.03
7.91	0.01 \pm 0.02
8.12	0.04 \pm 0.03

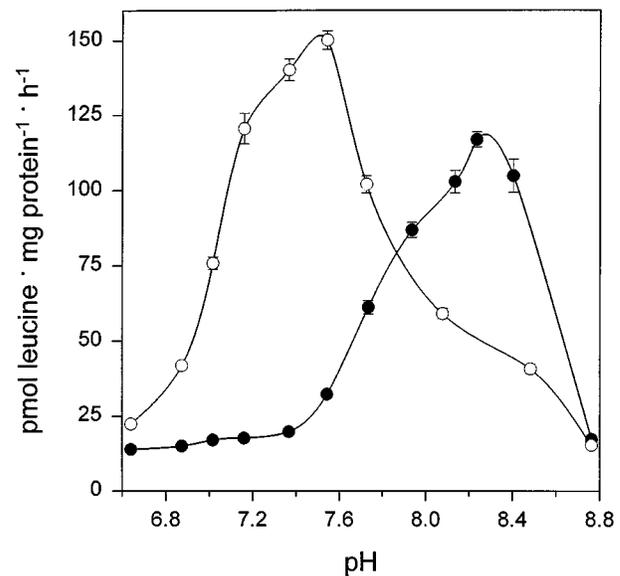


FIG. 5. Shift in the pH optimum for protein synthesis in the presence and the absence of nigericin in isolated mitochondria from *A. franciscana* embryos. Open symbols are assays performed in the absence of nigericin, and filled symbols are assays performed with 100 μ M nigericin. The data are the means \pm S.E. for three independent determinations at each pH.

oxidized (ferric) state (36), whereas antimycin A inhibits electron transport at the cytochrome *b-c* complex (site II). Within 2–3 min after the addition of either of these inhibitors, the redox state of the matrix was highly reduced and not significantly different from the anoxic treatment. Yet these inhibitors affected both the rate and extent of protein synthesis inhibition much less than did anoxia. Cyanide and anoxia have identical effects on cellular adenylate pools (37), so differences in the energy status are not a likely explanation. Consequently, additional factors must be operative under anoxia to depress protein synthesis to the degree observed.

The above data set is consistent with the presence of a molecular oxygen sensor within the mitochondrion that serves to mediate the rapid depression of protein synthesis observed under anoxia. Particularly relevant to this premise is the recent characterization of a cyanide- and antimycin-insensitive but hypoxia-sensitive form of cytochrome *b* that is apparently resident in the plasma membrane of carotid body cells and HepG2 cells. This cytochrome b_{558} is an integral component of the NAD(P)H oxidase and serves as an oxygen sensor that initiates an oxygen signaling cascade (38, 39). In our studies with isolated mitochondria, we gave special experimental attention to the difference between the hypoxia sensitivity and the cyanide/antimycin insensitivity of protein synthesis be-

cause it had previously been reported that the erythropoietin pathway in oxygen-sensing cells could be modulated by hypoxia but not by cyanide poisoning (40, 41) or by other respiratory chain inhibitors (42). The present work documents such an inhibitory signature for mitochondrial protein synthesis and now points to an oxygen-sensing mechanism located within this organelle.

It is appropriate to note that oxygen-linked byproducts could in principle explain the differential effect of anoxia and the inhibitors of the electron transport chain as well. Reactive oxygen species (e.g. superoxide free radical, H_2O_2) cannot be produced in the absence of oxygen but are generated aerobically by mitochondria (43, 44). The presence of electron transport inhibitors like antimycin A accentuate the leakage of single electrons from electron transport chains, thereby giving rise to univalent reductions of molecular oxygen and increased levels of reactive oxygen species (45, 46). Interestingly, specific protein sensors for both superoxide free radicals (SoxR) and H_2O_2 (OxyR) have been described in bacteria (47–50). The sensing mechanism of the SoxR protein involves the oxidation of an iron-sulfur center by the superoxide anion (50, 51); such a mechanism presumably would not be blocked by cyanide. The SoxRS regulon is also activated by the free radical nitric oxide (52), and nitric oxide synthase has recently been localized within the mitochondrion (53, 54).

In the well studied case of the yeast *Saccharomyces cerevisiae*, synthesis of mitochondrial-encoded subunits of cytochrome *c* oxidase is depressed in response to oxygen deprivation both *in vitro* (55, 56) and *in vivo* (reviewed in Refs. 16, 17, and 30). Translational regulation of mitochondrial-encoded subunits of cytochrome *c* oxidase in response to oxygen deprivation has been shown, in part, to be exerted by nuclear-encoded translational activators that bind to specific mitochondrial mRNAs and regulate the association with the small ribosomal subunit (reviewed in Ref. 16). As these activators become depleted within the mitochondrion during anoxia, protein synthesis is depressed (57). However, for *A. franciscana* embryos there is no direct evidence that nuclear-encoded proteins, whose expression is dependent on oxygen, influence rates of mitochondrial protein synthesis; protein synthesis rates under aerobic conditions are identical for mitochondria isolated from aerobic embryos compared with those of mitochondria isolated in the presence of cycloheximide from embryos exposed to 6 h of anoxia.² Rather, in *A. franciscana* mitochondria the available data suggest that the anoxia-induced depression of protein synthesis is globally mediated at the post-transcriptional level (13, 15).

Regulation of Mitochondrial Protein Synthesis by pH—As previously mentioned, if protein synthesis is sensitive to matrix pH, an alkaline shift in the extramitochondrial pH optimum for protein synthesis would be predicted when Δ pH is abolished. In the absence of nigericin, steady-state matrix pH was \sim 8.2 during protein synthesis assays performed at the optimal extramitochondrial pH of 7.5. With nigericin, the extramitochondrial pH optimum shifted to \sim 8.2 (Fig. 5). These data represent the first evidence that mitochondrial protein synthesis is sensitive to matrix pH and not directly to extramitochondrial pH. Furthermore, the small reduction in protein synthesis (\sim 20%) with nigericin indicates that Δ pH has little influence on mitochondrial protein synthesis. Recently, the opening of the permeability transition pore in the mitochondrion has been demonstrated by Bernardi and co-workers (58) to be acutely responsive to changes in matrix pH, such that low matrix pH

prevents pore opening. Not only does their work underscore another regulatory role for matrix pH, but it suggests a further reason why acidic pH during quiescence in *A. franciscana* embryos may be required for the extraordinary tolerance of these cells to anoxia (over two years; Ref. 4).

Although the pH sensitivity of protein synthesis has been noted in mitochondria from other organisms (e.g. rat heart (59), yeast (60), and mouse adrenal gland (61)), the pH effect on mitochondria from *A. franciscana* embryos is much more acute. The optimal pH range is narrower, and there is greater absolute difference between minimal and maximal rates over a similar range of pH. The data presented in this study indicate that there are proton-sensitive translational components within the mitochondrion. Thus, in conjunction with studies on the pH sensitivity of both catabolic (3, 5–7) and anabolic metabolism (9–11) in the cytoplasm of these embryos, our studies suggest that transitions in intracellular pH provide an intracellular signal integrating metabolic depression in both the mitochondrial and cytoplasmic compartments of *A. franciscana* embryos during transitions between active and anoxia-induced quiescent states.

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